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13. ABSTRACT (Maximum 200 Words)

The vast majority of fatal breast cancer cases involve deregulated cell proliferation and metastasis. The current project was originally funded to investigate and test the following hypothesis: Loss of activation of transcription factor Stat5 is a breast cancer progression event that favors epithelial-to-mesenchymal dedifferentiation, proliferation, invasiveness, and metastasis. However, upon completion of PI's coursework and comprehensive exams, she changed research labs within the same Tumor Biology Ph.D. training program, but more consistent with her overall breast cancer signal transduction educational and research objectivities. The laboratory switch entailed generation of new and promising pilot data, recently presented at the 2005 Lombardi Research Fair, and a requested revised statement of work. The requested revised statement of work focuses on investigation of the role calmodulin, a universal calcium sensor protein, in cell cycle progression of breast carcinoma. Based on literature and investigations from our lab, we now hypothesize that (1) calmodulin forms a tertiary complex with p55pik and Rb, and (2) activated calmodulin, by interacting with p55pik, phosphorylates Rb and induces cell cycle progression. We have developed the necessary reagents for the study and now aim to discover the nature of interaction between p55pik and CaM, and the role CaM plays in cell cycle regulation through p55pik. These studies could lead to new therapeutic drugs against breast cancer.

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Description of Research Project: The majority of fatalities from breast cancer involve the complications from deregulated proliferation, cell death, and metastases. However, the signal transduction pathways involved in these procedures are poorly understood.

The ***rationale*** of my original proposal centers on the role of Stat5 as a central suppressor of breast tumor dedifferentiation and progression. This was based on preliminary observations made in Dr. Hallgeir Rui's laboratory in the Lombardi Comprehensive Cancer Center. ***First***, Stat5 is constitutively active in normal human breast epithelium, and that this activation is lost during metastatic breast tumor progression. ***Second***, levels of active Stat5 in the primary tumor correlate positively with tumor differentiation. ***Third***, clinical data suggests loss of Stat5 activation in the primary tumor of patients with lymph node-negative breast cancer is associated with an eight-fold increased risk of latent, residual disease. My original goal was to establish the mechanism whereby Stat5 was a useful indicator of prognosis in lymph node negative breast cancer.

Original Statement of Work

Hypothesis: Loss of activation of transcription factor Stat5 is a cancer progression event that stimulates epithelial-to-mesenchymal dedifferentiation, which in turn leads to increased invasiveness and increased metastatic potential of breast cancer cells.

Aim #1: Establish the effect of activated Stat5 on homotypic adhesion, cell motility and invasion of human breast cancer cells, in vitro and in vivo.

Aim#2: Determine the invasion-regulatory effect of activated Stat5 on established human breast tumors in nude mice using adenoviral delivery.

Aim #3: Determine the effect of Stat5 activation on invasion and metastasis of mouse breast cancer models in vivo.

Unexpected Difficulties Encountered and Deviations from the Original Statement of Work

During the past year, I changed labs from that of Dr Hallgeir Rui to Dr. Robert B. Dickson. Dr. Robert B. Dickson was an original co-mentor of mine for the initially approved statement of work. He directs Lombardi Comprehensive Cancer Center Tumor Biology Ph.D. Program and has conducted research in breast cancer for more than 20 years. He has published almost 200 publications in the field of breast cancer research, and his laboratory affords the opportunity for me to fully accomplish my overall educational objectives to facilitate the beginning of my career in breast cancer research. However, this change of lab made it unrealistic for me to continue working specifically on transcription factor Stat5; however, I now have the opportunity to work on a project about a different signal transduction pathway involves human breast cancer. The deviation from the original statement of work will be stated in the following requested revised statement of work.

Revised Statement of Work

Proposal Body

Career/Research Plans: My predoctoral training in the laboratory of Dr. Robert B. Dickson, PhD, Professor of Oncology, Lombardi Comprehensive Cancer Center (LCCC) at Georgetown University Medical Center will be very thorough and will facilitate my path to a successful career as an independent scientist in breast cancer research. The laboratory is very well positioned technologically for novel studies of the role of calcium/calmodulin in progression of breast cancer. Opportunities within the laboratory are excellent for training in experimental manipulation of signal transduction pathways by gene delivery by nucleofection, antisense strategies, and inhibitors. Advanced DNA manipulation techniques, protein analysis by biochemical methods, immunoblotting and immunohistochemistry are also in routine use.

Within the LCCC, I can take advantage of additional training opportunities offered by the institutional core facilities, such as transgenic mice, cell culture facility, microscopy and imaging facility, and macromolecular analyses facility. In addition to Tumor Biology classes, I will be closely involved with the LCCC weekly seminar series in Tumor Biology, as well as weekly Tumor Biology data meeting and journal club presentations in breast cancer research. In addition, my lab meets weekly for data presentation and discusses relevant breast cancer papers from the literature. I have already registered for the 2005 DOD Era of Hope meeting to present my data. I will also attend and present data at international meetings annually, such as AACR, Keystone symposia in Breast and Prostate Cancer, and Gordon Conferences.

Upon completion of this project, I plan to apply for postdoctoral training in another advanced laboratory where I can gain insight into onset and progression factor analysis of breast cancer. I believe that early detection may have the most significant positive impact on breast cancer prognosis and cure. By combining the molecular knowledge of onset and progression of breast cancer from my predoctoral training with additional insight into this field, I envision my future laboratory focusing on improving early diagnosis and elimination of breast cancer. With these training experiences, I will be well prepared for a career as a successful Principal Investigator working at the cutting edge of molecular breast cancer research, with the goal of directly and positively impact diagnosis and treatment of breast cancer patients.

Description of Research Project: **Calmodulin (CaM)** is a ubiquitous, calcium-binding protein that senses the cellular calcium level and relays a Ca^{++} signal to multiple protein targets. Calmodulin also binds to and relays the growth factor-dependent signals from epithelial growth factor receptors, such as the EGF receptor. The Dickson laboratory has studied the pathophysiologic roles of EGF receptor family signaling for many years. Recently, the investigations in our lab have demonstrated that growth factor stimulated CaM activation can result in phosphorylation and activation of Akt in c-Myc overexpressing mouse mammary carcinoma cells (Deb et al., 2004). We also found that CaM associates with a PI3 Kinase regulatory subunit, **p55pik**, in mouse and human

breast cancer cell lysates. P55pik is thought to be involved in cell cycle regulation by binding to Rb through its N-terminal 24 amino acids (Xia, et al., 2003). Overexpression of the N-terminal 24 amino acids of p55pik in breast cancer cells leads to cell cycle arrest (Xia, et al., 2003). However, the specific role played by CaM-p55pik interaction in cell cycle regulation remains unclear. To address this issue, we will investigate **the nature of interaction between p55pik and CaM, and the role CaM plays in cell cycle regulation through p55pik**. This project will provide insight into a novel partner of CaM in cell cycle regulation, and towards identification of a new possible anti-breast cancer proliferation drugs.

Requested Aims for New Statement of Work
Specific aims:
***Hypothesis 1*: P55pik directly associates with both CaM and Rb, forming a tertiary complex.**
Specific aim 1. Determine if Sf9-generated p55pik selectively binds CaM in the presence of calcium.
Specific aim 2. Investigate using microscopic live images, if p55pik binds CaM in the erbB2/HER2 receptor-expressing AU565 and SKBr3 breast cancer cells.

***Hypothesis 2:* Activated calmodulin, by binding p55pik, phosphorylates Rb and causes cell cycle progression.**
Specific aim 3. Determine if overexpression of p55pik results in cell cycle progression in breast cancer cells with activated CaM.
Specific aim 4. Determine if knockdown of p55pik protein expression by RNA interference leads to cell cycle arrest in breast cancer cells with activated CaM.

Background and significance
Introduction:
Breast cancer is a malignant tumor that begins in the ducts, lobules, or stroma of the breast structure. Breast cancer is the second leading cause of cancer death in woman, one out of seven women is expected to develop invasive breast cancer at some time in their lives (www.cancer.org). The American Cancer Society has estimated that in 2005, about 211,240 new cases of invasive breast cancer will be diagnosed in US, leading to 40,410 deaths (www.cancer.org). In the US population alone, current numbers of diagnosed breast cancer patients are more than two million (www.cancer.org). In the past several decades, although progress has been made in early diagnosis and treatment of breast cancer, mechanisms for the onset and progression of human breast cancer remained poorly understood.

Calmodulin
Two of the fundamental etiologic processes in tumorigenesis are that cancer cells develop a way to evade apoptosis and proceed with deregulated cell cycle progression (Hanahan, et al., 2000). CaM facilities phosphorylation of serine/threonine kinase Akt, a central player controlling cell survival (Dudek et al., 1997; Kauffmann-Zeh et al., 1997) through CaM-dependent protein kinase kinase (Yano et al., 1998). A new, potentially significant role played by calcium/CaM in proliferation/survival of mammary epithelial cells has

gradually been recognized. It was reported that mammary epithelial cells release abundant calcium from intracellular reservoir upon growth and survival factor stimulation (Ichikawa et al., 2000, 2001). Recently, a transgenic mouse model with targeted CaM overexpression in the heart confirmed that excess CaM enhances the extent of DNA synthesis and cell proliferation, resulting in ventricular hypertrophy (Colomer et al., 2004). A recent investigation provided physical evidence for growth factor-dependent CaM activation by identification of CaM as a binding protein for Epidermal Growth Factor Receptor (EGFR) and ErbB2/Neu/Her2 receptor (Li et al., 2004). The activation of an important lipid second messenger generator, PI-3Kinase, is dependent on Calcium/CaM for production for phagosome maturation *in vivo* in a newly discovered hVPS34 cascade (Vergne et al., 2003). It was previously reported that CaM is overexpressed in human breast cancer cell line *in vitro* (Chun & Sacks, 2000), as well as in primary tumors of breast cancer patients (Desai et al., 2002). Investigations in our lab have revealed a novel, calcium/CaM-dependent Akt activation and cell survival mechanism in c-Myc overexpressing mouse mammary carcinoma cells (Deb et al., 2004). This mechanism seems to involve transportation of Akt to the plasma membrane, where its phosphorylation and activation occurs.

P55pik (p55 gamma) and Retinoblastoma protein (Rb)

PI-3Kinase is implicated in the regulation of diverse cellular processes, including cell transformation, cell cycle progression, and anti-apoptosis. P55pik is a regulatory subunit of PI-3 kinase that is expressed in mammary epithelial cells and breast cancer cells (Xia et al., 1999; Pons et al., 1995). It has a unique 30 amino acid NH2 terminus, with a function not completely clear, while significantly, its C-terminal SH2 domains are almost identical to those in p85 regulatory subunit of PI-3Kinase (Pons et al., 1995) (Fig.1). Recently, p55pik has been shown to play a role in cell cycle progression through its interaction with Rb, a key regulator of cell cycle progression (Xia et al., 2003). In quiescent breast cancer cells, the N-terminal 24 amino acids of p55pik bind to hypophosphorylated Rb, leading to the G0/G1 phase cell cycle arrest, inhibition of DNA synthesis and cyclin D/E promoter activity (Xia et al., 2003). Cell cycle progression was observed following growth factor-induced Rb phosphorylation and release of hyperphosphorylated Rb from p55pik (Xia et al., 2003).

The retinoblastoma (*rb*) gene was the first tumor suppressor gene, identified by its involvement in hereditary retinoblastoma. Accumulating evidence from *in vitro* and *in vivo* studies have confirmed that its gene product, a 105 KDa protein, is implicated in cellular functions including cell proliferation, differentiation, and apoptosis (Classon et al., 2002). The major function of Rb is as a cell cycle inhibitor; cells undergo arrest in the G1 phase of the cell cycle upon the activation of Rb. Rb directly binds and inhibits the transcriptional activity of E2F family members by recruitment of several chromatin-remodeling complexes to promoter regions, and results in chromatin condensation and transcription inhibition (Harbour et al., 2001). The function of Rb depends on its phosphorylation status. The underphosphorylated Rb is the active form, which has growth suppressive activity, and phosphorylated Rb is inactive and unable to bind E2F transcription factors (Goodrich et al., 1991).

Significance

Our investigation will clarify the role of P55pik-calmodulin interaction in Rb phosphorylation and cell cycle progression of tumor cells. Since calmodulin is a ubiquitous protein in all cells of vertebrates, and since inactivation of this protein will be fatal to the whole living system, defining a new Rb interacting protein that involves in role of CaM in cell cycle regulation will benefit our understanding of cancer etiology and possibly towards cancer therapy.

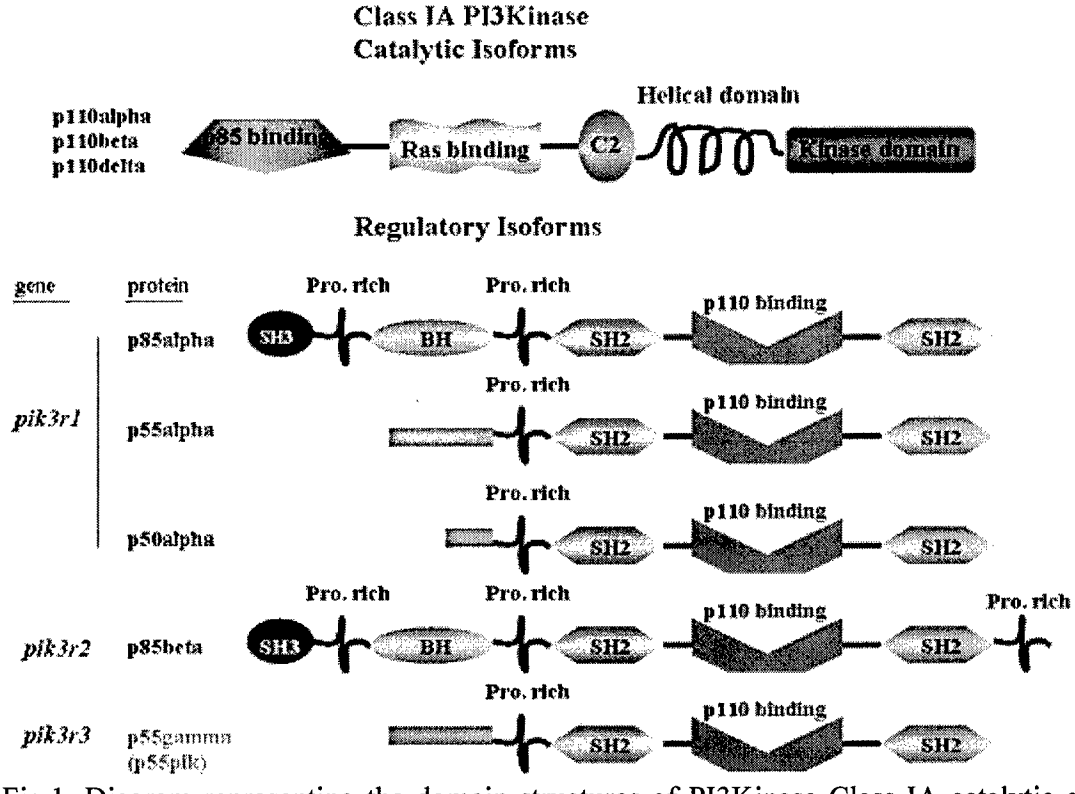

Fig.1. Diagram representing the domain structures of PI3Kinase Class IA catalytic and regulatory isoforms. P55pik, also named p55gamma, is encoded by a distinct gene, *pik3r3*. (Adapted with modification from Donahue et al., 2004)

Preliminary Results (All experiments conducted by myself)

Analysis of potential CaM binding sites on p55pik

The Calmodulin Target Database (Yap et al., 2000) was used for computer analysis of the p55pik protein sequence. The criteria used in this analysis include hydropathy, alpha-helical propensity, residue weight, residue charge, hydrophobic residue content, helical class, and occurrence of particular residues. In the p55pik sequence, the most likely binding sites are located at amino acid residues 102-114, LTLRKGGNNKLIK (Fig.2). This binding site analysis indicates that a CaM-binding site on p55pik is located in the Src Homology 2 (SH2) domains, like other regulatory units in PI-3Kinase family, such as p85 (Joyal et al., 1997).

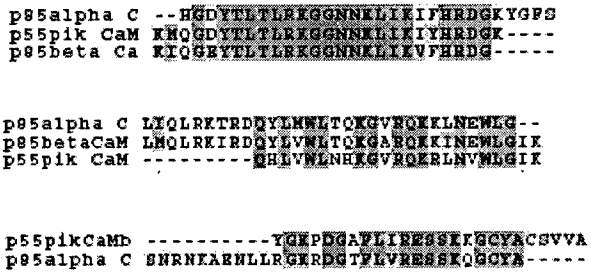

Fig.2. The potential CaM binding site on human p55pik protein sequence is conserved among the regulatory subunits including p85alpha and p85 beta. All match regions were highlighted in green; yellow shade indicates conserved regions, and similar regions were in cyan.

Association between p55pik and CaM in AU565 breast cancer cell lysates

Treatment of AU565 breast cancer cell lysates with or without calcium, or with calcium plus chelator showed a calcium-dependent CaM-p55pik association (Fig. 3).

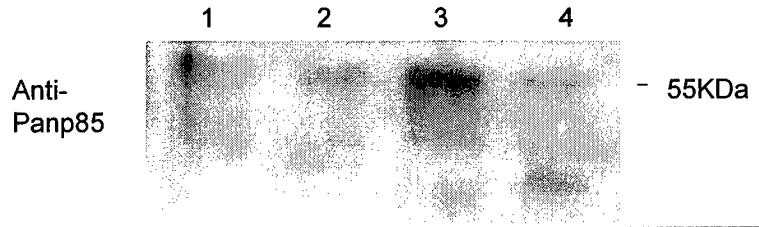

Fig.3. Calmodulin specifically binds to p55pik in a calcium-dependent manner. AU565 cells were serum-starved, cell lysates were incubated with 50 µl of calmodulin-sepharose beads in the absence (Lane 2), or in the presence (lane 3) of 0.1mM $CaCl_2$ and 1mM EGTA plus 0.1mM $CaCl_2$ (lane 4) for 2 hrs. Lysates incubated with non-conjugated sepharose were used as a negative control (lane 1). Washed beads were resolved by SDS-PAGE and transferred to PVDF membrane. Blots were probed with anti-p85pan pAb from Santa Cluz Biotechnology (CA), which recognizes p85alpha, p85beta, and p55pik, for PI-3Kinase subunits. Data are representative of two independent experimental determinations.

Estimation of the titer for rabbit antisera against N-terminal 18 amino acids of p55pik (anti-Np55pik) with antigenic synthetic peptide

We incubated sera collected from rabbits before and after DNA immunization with the peptide. The immunized rabbit anti-sera showed specificity for synthetic peptide and did not cross-react with BSA. The normal rabbit sera did not bind peptide (Fig .4).

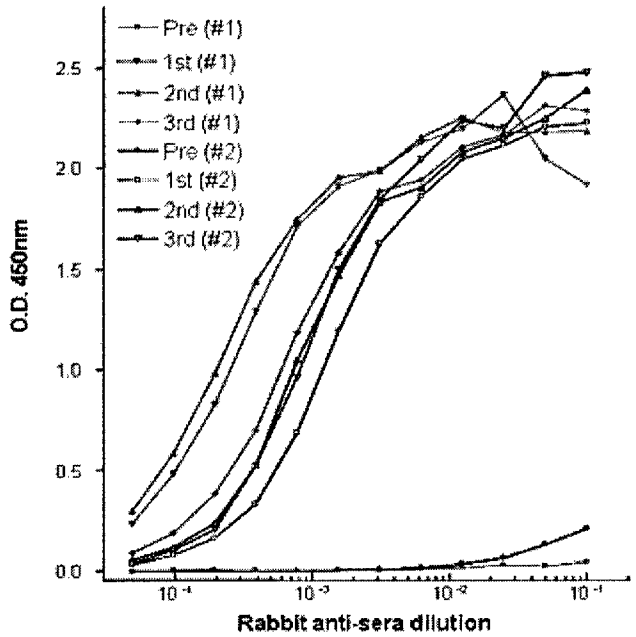

Fig.4. NP55pik anti-sera demonstrated specificity towards the synthetic peptide by ELISA. Ninety-six-well microtiter plates were coated with 0.5 μg/ml synthetic peptide in 50 mM bicarbonate buffer (pH9.5) at 4°C overnight, blocked with 1% of BSA in PBS at room temperature for 1 hr, and washed with PBS containing 0.05% Tween 20 (PBST). Rabbit serum (50 μl) diluted 1:100 in PBST containing 1% BSA, were added to pre-coated wells. After 1 hr at room temperature, the plates were washed four times. Each well was then incubated for 1 hr with 50 μl of a 1:6000 dilution of goat IgG F(ab')2 anti-rabbit labeled with horseradish peroxidase (HRP) at room temperature. Assays were developed with TMB/H_2O_2 (tetramethyl benzidine) substrate. The absorbance was read at 450 nm.

Purification of anti-Np55pik rabbit antisera with peptide-conjugated affinity column

Five mg of peptide was coupled to a 1 ml affinity column with 5 hrs incubation at room temperature. Buffer A (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3) and Buffer B (0.1 M acetate, 0.5 M NaCl, pH 4) were alternatively applied to deactivate any excess active groups that have not coupled to the ligand and wash out the non-specific binding ligand. Then 30ml of rabbit antisera diluted in 70 ml of PBS were clarified by passing through a 0.45μm filter and injected into the equilibrated column at a flow rate of 1 ml/min, washed with 10ml of PBS, and eluted with 3ml of elution buffer (50 mM Citric Acid, phosphate buffer, pH 3.4).

Experimental Design

Specific aim 1. Determine if Sf9-generated p55pik binds CaM in the presence of calcium.

Specific aim 2. Investigate using microscopic live images, if p55pik binds CaM in the erbB2/HER2 receptor-expressing AU565 and SKBr3 breast cancer cells.

Rational for Aim 1 and 2. Immunoprecipitation experiments have suggested the association between p55pik and calmodulin. Using a computer search, putative CaM binding sites have been identified in the SH2 domain of p55pik sequence, which are conserved among p55pik, p85α, and p85β. Furthermore, literature indicated a direct, calcium-dependent binding between SH2 domain of p85 and CaM (Joyal et al., 1997). These two lines of evidences therefore enable us to propose that CaM also interact directly with the SH2 domain of p55pik.

Methods

1. Direct binding assay in cell lysates: Pilot studies demonstrated that bacterial production of GST-P55pik using prokaryotic expression vector pGEX-4T-1 was insufficient for *in vitro* assay. Therefore, I will generate GST-p55pik protein produced following baculovirus infection from Sf9 cells. Cell lysates will be purified with glutathione-sepharose beads. Purified GST-p55pik protein will be passed through a CaM sepharose column in the presence of Ca^{++}. The column will be eluted by EGTA, and both bound and unbound fractions will be assayed for p55pik protein by western blotting.

Expected results and backup strategies: I expect that the majority of p55pik protein input will be detected in the bound fraction, with only a small fraction of input washed through CaM column. The concentration of Ca^{++} that is sufficient for activation of CaM and protein-protein interaction is a parameter to be determined. I also need to experiment on the molar ratio between p55pik and CaM, for an optimized interaction. Alternatively, if the direct interaction between p55pik and CaM cannot be confirmed by this assay, I will use a yeast-two hybrid assay to test my aim and to potentially find new p55pik-interacting proteins.

Yeast-two-hybrid screening assay: I will use the commercially available BD Matchmaker™ Two-Hybrid System 3 (Clontech), following the mamnufacturer's protocol. A cDNA expression library will be prepared from human breast cancer AU565 cells, in cloning vector pGADT7, as the prey. Since I propose that p55pik is the bridge between CaM and Rb, and the interacting proteins of p55pik are largely unknown, p55pik cDNA fused to the DNA binding domain of GAL4 will be used as the bait plasmid. The results of this assay will be confirmed by GST-pull down assay. I anticipate many cell signaling proteins as well as membrane receptors will be picked out, such as CaM, Rb, IRS-1, IGF-1R and insulin receptor. While investigation of protein-protein interaction using a human-origin protein library expressed in yeast is a method with broad application, the difference between prokaryotic and eukaryotic expression systems can not be neglected. Due to the lack of posttranslational modifications, such as phosphorylation, acetylation, isoprenylation, glycoslyation, and GPI anchor etc., I expect that many false positive colonies will blend in, and not all of the negative colonies would be real negatives.

2. Direct binding assay in living cells: In order to examine the p55pik and CaM interaction in living cells, I will use the Fluorescence Resonance Energy Transfer (FRET) method. FRET refers to the nonradiactive transfer of energy between two fluorophores, where the emission of one fluorophore, namely the donor, overlaps dramatically with the other, the acceptor. The occurrence of FRET is largely dependent on the distance between the fluorophores as well as their dipole-dipole orientantions. When studying the

interaction between fluorescence labeled cellular components, FRET based analysis can provide more specific and higher spatial resolution than conventional light microscopes.
Design of FRET: My studies will use a particularly robust FRET pair; a functional calmodulin-EYFP (Enhanced Yellow Flurescent Protein) chimera will be used as the acceptor fluorophore, and a p55pik-ECFP (Enhanced Cyan Flurescent Protein) chimera will serve as the donor fluorophore (Jobin et al., 2003). Fluorescent construct pairs will be transiently and stably expressed in AU565 and SKBr3 cells for interaction studies. First, I will confirm the formation of CaM and p55pik complexes by immunoprecipitation. CaM-p55pik interaction will be tested in extracts from cells treated, with or without EGF, and with or without 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl)ester (BAPTA-AM), a permeable free calcium chelator. Then, the calmodulin- EYFP and p55pik-ECFP coexpressing cells will be analyzed with fluorometry, video-microscopy and confocal microscopy, using an instrument from Photon Technologies Inc. (Lawrenceville, NJ), available in the LCCC microscopy and Imaging Facility.
Expected results and backup strategies: I anticipate that using acceptor photobleaching techniques, with morphometric analysis, we should be able to demonstrate a direct, calcium dependent binding between calmodulin and p55pik. If I am unable to show the direct interaction between them *in vivo*, then I will employ site directed mutagenesis of CaM binding sites on p55pik, selected potential binding sites will be deleted or mutated, possible interruption of CaM-p55pik will be examined by co-transfection of plasmids containing mutant and CaM cDNA and immunoprecipitation.
Significance: This part of the project will identify the direct interaction between p55pik and CaM. Identification of direct interacting proteins provides a helpful hint in determining the potential biological functions of p55pik, it may also lead to the clarification of mechanisms that we can manipulate to interfere with cell growth and cell cycle progression in cancer cells.

Specific aim 3. Determine if overexpression of p55pik results in cell cycle progression in breast cancer cells with activated CaM.
Rational for Specific aim 3: CaM-sepharose pull-down assays in our lab suggested the interaction between CaM and p55pik exists in mouse mammary carcinoma cells Mayc83 (Deb T, unpublished data) and human breast cancer AU565 cells (Wang Y, unpublished data). The function of p55pik, although still not clear, should be associated with CaM activation. In quiescent breast cancer cells, the N-terminal 24 amino acids of p55pik bind to hypophosphorylated Rb, leading to G0/G1 phase cell cycle arrest, inhibition of DNA synthesis, and cyclin D/E promoter activity (Xia et al., 2003). Cell cycle progression was observed following growth factor-induced Rb phosphorylation and release of hyperphosphorylated Rb from p55pik (Xia et al., 2003). We believe the linkage between these observations positions p55pik, a regulatory subunit of PI3Kinase, in a tertiary complex comprising CaM and Rb. We propose that activated CaM binds to p55pik and leads to increased phosphorylation of Rb. Overexpression of p55pik will result in hyperphosphorylation of Rb and cell cycle progression in Rb positive cell lines, such as SKBr3, AU565, and MCF-7.

Methods

P55pik overexpression in Rb positive, growth factor-receptor positive cells

Experiment Design: SKBr3, AU565, and MCF-7 cells will be transfected with plasmid pCDNA3.1, containing the cDNA of human p55pik. Empty vector pCDNA3.1 will be transfected as a negative control. Stable expressing cell lines will be established using the G418 selection method, over a period of four weeks. Positive clones, containing high copy numbers of recombinant plasmid, will be selected by screening p55pik protein expression with purified anti-Np55pik antibodies. The p55pik-high-expressing clones will be pooled and reduced to quiescent stage by continuous culturing in growth medium containing 0.1~0.2% FBS. Next, these quiescent cells will be stimulated with EGF for 24 hrs, before being subjected to flow cytometry analysis. Flow cytometry will determine the cell cycle progression profile of each individual cell line. The nuclear DNA stain, propidium iodide (PI), will be used to directly discriminate cells at different stages of the cell cycle. Three histogram markers distinguish the G0/G1, S and G2/M cell cycle phases, while a fourth marker offers a way to identify apoptotic cells, cell aggregates, or an internal standard (Guava Technologies. Inc., CA). Cell lysates will be resolved and detected for Rb phosphorylation with 3 commercial available anti-phospho Rb antibodies (Cell Signaling, Beverly, MA).

Expected Results: I expect that different levels of Rb phosphorylation will be observed, comparing empty vector and recombinant plasmid stable cell lines, with more phosphorylated Rb in p55pik-overexpressing groups. The cell cycle profile will reveal smaller percentages of cells existing in the G0/G1 stage in the p55pik-overexpressing groups than control groups.

Significance: Establishing a breast cancer cell line overexpressing human p55pik will help us understand the biological functions of p55pik, in terms of cell growth and cell survival. If induction of the cell cycle progression can be confirmed in the stable cell lines, we can further test their tumorigenic properties in animal studies.

Specific aim 4. Determine if knockdown of p55pik protein expression by RNA interference leads to cell cycle arrest in breast cancer cells with activated CaM.

Rational for aim 4. Interfering with the expression or function of p55pik protein should have a negative effect on the signal transduction between CaM and Rb. This negative effect, if strong and sufficient, will lead to cell cycle arrest, even when activation of CaM is induced by growth factors.

Methods

Knocking down p55pik protein expression with Small interfering RNA. Four pairs of Small interfering RNAs directed against *pik3r3* were designed and synthesized at Dharmacon, Inc (Chicago, IL). P55pik SiRNA will be delivered into MCF-7 and AU565 cells, with SiRNA against Luciferase delivered into parallel groups as a non-target control, and a negative control SiRNA. 48 hours after transfection, cells will be serum-starved overnight and stimulated with EGF the next day. The interference of p55pik protein expression will be monitored by specific anti N-terminal p55pik antibody. Rb

phosphorylation status will be detected by phospho Rb antibody. The apoptosis status of the cells will be detected using Apo-ONE Homogeneous Caspase-3/7 Assay by Promega Corporation (Madison, WI).

Expected results: I expect to achieve at least 75% knockdown of the p55pik protein expression compared to the negative control group, in both MCF-7 and AU565 cells. The degree of Rb phosphorylation will determine whether p55pik is involved in the hypophosphorylation-to-hyperphosphorylation transition of Rb. Cell cycle arrest is expected to lead to more apoptotic cells in the P55pik knockdown group than control group.

Significance: Discovery of a functional p55pik-CaM interaction that leads to Rb phosphorylation and cell cycle progression will identify p55pik as a protein that plays important role in cell proliferation. This will possibly establish a foundation for the development of a new anti-cancer drug that works synergistically with other drugs against human breast cancer.

Statement of Work

Interaction between P55pik and Calmodulin, and its biological implications in Cell Cycle Regulation

Task 1) Determine if Sf9-generated p55pik binds CaM in the presence of calcium. (months 1-12)

a. Generate viral constructs for GST-p55pik protein expression. (months 1-2)
b. Transfect the insect Sf9 cells with plasmid-containing baculovirus, analysis of the transfected cells with anti-GST and anti-Np55pik antibodies for protein expression. (months 3-5)
c. Purify the verified GST-p55pik recombinant protein with glutathione-sepharose beads. (months 6-7)
d. Pass the purified protein through a CaM sepharose column in the presence of Ca^{++}, elute the column and assay the fractions by western blotting. (months 8-12)

Task 2) Investigate using microscopic live image, if p55pik binds CaM in the erbB2/HER2 receptor-expressing AU565 and SKBr3 breast cancer cells. (months 1-24)

a. Generate eCFP, or eYFP expressing constructs containing cDNA of p55pik, and CaM, respectively. (months 1-4)
b. Transient transfect AU565 and SKBr3 cells with the constructs and analysis of the protein expression as well as the protein biological activities. (months 5-8)
c. Generate AU565 or SKBr3 cell lines stable expressing both CaM-eYFP, and p55pik-eCFP recombinant proteins (months 9-12)
d. Assay the protein-protein interaction using FRET with fluorometry, video-microscopy and confocal microscopy. (months 13-24)

Task 3) Determine if overexpression of p55pik results in cell cycle progression in breast cancer cells with activated CaM. (months 1-18)

a. Transfect SKBr3, AU565, and MCF-7 cells with plasmid pCDNA3.1, containing the cDNA of human p55pik. Select stable expressing cell lines using the G418 selection method. (months 1-3)
b. Screen positive clones with anti-Np55pik antibodies. (months 4-5)
c. Pool the positive clones and synchronize the cells, stimulate cells with EGF and analyze cell cycle progression profile with flow cytometry (months 6-12)
d. Detect the activation status of Rb in these cell lysates using anti-phospho Rb antibodies (Cell Signaling, Beverly, MA). (months 13-18)

Task 4) Determine if knockdown of p55pik protein expression by RNA interference leads to cell cycle arrest in breast cancer cells with activated CaM. (months 1-24)

a. Pilot study for the optimal transfection efficiency of four individual SiRNAs against p55pik using real-time PCR and anti-Np55pik antibodies. (months 1-6)
b. Transfect the selected SiRNAs into AU565 cells and analyze the interference of p55pik protein expression, Rb phosphorylation status, and the apoptosis status of the cells. (months 7-24)

Publication of this work to date
ABSTRACTS presented

1) Abstract # 62 Identification of Human P55pik Interacting Proteins: Production and Characterization of P55pik Recombinant Proteins and Antibodies. 7th Annual Lombardi Research Fair at Georgetown University Lombardi Comprehensive Cancer Center. February 9-11, 2005

2) Towards identification of human p55pik interacting proteins: production and characterization of p55pik recombinant proteins and antibodies. Youhong Wang, Tushar Deb, and Robert Dickson. Department of Defense Breast Cancer Research Program (DOD, BCRP) Annual Meeting, the 4th Era of Hope in Philadelphia, Pennsylvania. June 8-11, 2005

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1) Identification of Human P55pik Interacting Proteins: Production and Characterization of P55pik Recombinant Proteins and Antibodies.

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Insulin-like growth factor (IGF)-1 is one of the most potent mitogens to breast cancer cells. A majority of breast cancers express the IGF-1 receptor. Among the proteins along the IGF-axis, p55pik plays a special role as a regulatory subunit of phosphotidylinositol-3 kinase (PI3K). The mRNA level of p55pik is higher in estrogen receptor positive than negative breast cancer cells, although its pathophysiologic function is unknown. In order to explore biological functions of p55pik, it is necessary to identify its interacting proteins. Towards this goal, we are beginning to establish an immunoprecipitation system by generating recombinant p55pik protein and p55pik-specific antibodies.

Methods

- A *E.coli.* Mammalian Gene Collection (MGC) clone containing full-length p55pik cDNA was purchased from Invitrogen. P55pik cDNA was cloned to construct eukaryotic expression plasmids, either with or without a tag (to produce Flag or EGFP protein) for transfections.

- Plasmids containing p55pik were transfected into three mammalian cell lines (MCF-7, 293T and COS-7). MCF-7 cell lines overexpressing p55pik were screened by G418.

- Two prokyrotic expression constructs were designed and used to produce p55pik-GST fusion and MBP fusion proteins.

- SDS-PAGE, Western blots, and flow cytometry were performed to characterize their expression.

- An 18 amino acid peptide antigen (DDADWREVMMPYSTELIF) derived from the N-terminal region of p55pik, which is unique to p55pik in the PI3K family, was designed and synthesized.

- Rabbit antisera were collected monthly during the four-month period with three times of immunizations.

- ELISA was used to determine the titers (1:10,000) of antibodies specific for p55pik after the last booster.

- The specific antibodies (IgG) were isolated by affinity chromatography using a protein-A column.

Conclusions

In the present work, we have successfully constructed and expressed recombinant p55pik proteins and established cell lines overexpressing p55pik. We also produced rabbit antibodies specific for the N-terminal of p55pik. This work has established a foundation for further identification of the p55pik-associated proteins, which would help us understand the biological functions of p55pik. More importantly, it will provide new molecular targets for breast cancer therapy.

Future Directions
To identify p55pik associated proteins by immunoprecipitation using purified p55 GST fusion proteins, anti-GST antibody or antibodies specific for the N terminal of p55pik, and the new MCF7.p55 cell line.

2) Towards indentification of human p55pik interacting proteins: production and characterization of p55pik recombinant proteins and antibodies.

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Insulin-like growth factor (IGF)-1 is one of the most potent mitogens to breast cancer cells. A majority of breast cancers express the IGF-1 receptor. Among the proteins along the IGF-axis, p55pik plays a special role as a regulatory subunit of phosphotidylinositol-3 kinase (PI3K). Although its pathophysiologic function is unknown, the mRNA level of p55pik is higher in estrogen receptor positive than negative breast cancer cells. In order to explore biological functions of p55pik, it is necessary to identify its interacting proteins. Towards this goal, we are beginning to establish an immunoprecipitation system by generating recombinant p55pik protein and p55pik-specific antibodies.

We constructed three different eukyrotic expression plasmids containing P55pik, either with or without a tag (to produce Flag or EGFP protein) for transfections, and expressed p55pik in three mammalian cell lines (MCF-7, 293T and COS-7). Two prokyrotic expression constructs were designed and used to produce p55pik-GST fusion and MBP fusion proteins. SDS-PAGE, Western blots, and flow cytometry were performed to characterize their expression. We also designed an 18 amino acid peptide antigen (DDADWREVMMPYSTELIF) derived from the N-terminal region of p55pik, which is unique to p55pik in the PI3K family. Rabbit antisera were collected monthly during the four-month period with three times of immunizations. ELISA was used to determine the titers (1:10,000) of antibodies specific for p55pik after the last booster. The specific antibodies (IgG) were isolated by affinity chromatography using a protein-A column.

We successfully expressed the IPTG-inducible GST-p55pik fusion protein in BL21(DE3) bacteria. The SDS-PAGE of IPTG induced bacteria lysates revealed a protein band of about 82 KDa, which is consistent with the theoretical molecular mass of GST protein (27Kda) combined with p55pik (55KDa). We also expressed EGFP-p55pik fusion proteins in MCF-7 and obtained a cell line stably expressing P55pik. Immunoblot analysis of cell lysates of MCF-7 and COS-7 showed a specific band at 82 KDa

recognized by the anti-GFP monoclonal antibody. The molecular mass is consistent with the theoretical molecular mass of GFP (27KDa) combined with p55pik. The expression was also confirmed by flow cytometry, using an anti-GFP antibody. In addition, the p55pik-specific rabbit antiserum was purified by affinity chromatography. We found that the p55pik-specific polyclonal antibody has no cross-reaction with p85 proteins in the same PI3K family. Interestingly, our preliminary experiments suggested that calmodulin (CaM) might be a protein interacting with p55pik in both mouse and human breast cancer cells.

In the present work, we have successfully constructed and expressed recombinant p55pik proteins and established cell lines overexpressing p55pik. We also produced rabbit antibodies specific for the N-terminal of p55pik. This work has established a foundation for further identification of the p55pik-associated proteins, which would help us understand the biological functions of p55pik. More importantly, it will provide new molecular targets for breast cancer therapy.

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